Int J Biol Med Res. 2010; 1(4): 228-233

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original article

Therapeutic Potential of *C. zeylanicum* Extracts: An Antifungal and Antioxidant Perspective

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ARTICLE INFO

Keywords:
Antifungal
Antioxidant
Cinnamomum zeylanicum
Extracts
MFC
Reducing power assay

ABSTRACT

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. These secondary metabolites could be utilized for benefit of mankind by studying their medicinal properties. Present work reports the antifungal and antioxidant activities of various bioactive fractions extracted from bark and leaves of *Cinnamomum zeylanicum*. Fungicidal activity of the extracts was evaluated against pathogenic and spoilage fungi, namely, *Aspergillus flavus, A. fumigatus, A. niger, Penicillium spp. and Candida albicans.* Many extract fractions derived from cinnamon samples exhibited low to moderate fungicidal activities. Polar fractions demonstrated comparatively better responses. Minimum fungicidal concentration (MFC) was found in the range 300-1000 µg/ml. The antioxidant activities of the fractions were evaluated by using reducing power assay and the results were compared with standard antioxidant ascorbic acid. Aqueous, ethanolic and acetone fractions showed appreciable reducing power. None of the fractions exerted pro-oxidant activity. The antioxidant activity increased with increasing amount of the extracts showing dose dependent response. The fungicidal and antioxidant activities may be attributed to the presence of several secondary metabolites such as phenolic and flavonoid compounds present in the extract fractions. The results obtained in the present study indicate that the bark and leaves of *C. zeylanicum* are potential sources of natural antimicrobial and antioxidant compounds.

1. Introduction

Plant extracts have been known since antiquity to possess notable biological activities, including antioxidant, antibacterial, and antifungal properties. There is a growing interest in the use of natural products in the human food and animal feed industries as consumer resistance to synthetic additives is increasing [1]. Antimicrobial properties of herbs and spices have been used since time immemorial for food preservation and medicinal purposes [2]. Fungi cause major destruction of various food commodities during storage. Further the production of mycotoxins by them constitutes a serious threat to human health. Aflatoxin and ochratoxin produced by species within the genera *Aspergillus* and *Penicillium* are most toxic to mammals, causing a variety of adverse effects including hepatotoxicity, teratogenicity and mutagenicity, resulting in diseases such as toxic hepatitis, hemorrhage, oedema, immunosuppression, hepatic carcinoma, equine leukoencephalomalacia (LEM), esophageal cancer and kidney failure [3]. *A. niger* is an opportunistic human pathogen and a strong air pollutant. The other common pathogenic species *A. fumigatus and A. flavus* produce toxicity and carcinogenicity. *Candida albicans*, a dimorphic fungus, causes a variety of superficial and deep-seated mycoses [4].

Many synthetic fungicides have been used to overcome the destruction of food products. The drawback related with use of synthetic fungicides is that they enter into food chain and thus constitute pesticide pollution as well as several side ill effects. Recently some higher plants and their constituents have been reported as an ideal natural fungitoxicants in controlling plant diseases because of their lesser phytotoxicity, more systemisity and easily biodegradable nature [5]. Numerous studies have documented the antifungal and antibacterial effects of plant extracts and essential oils [6-11].

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Oxidative stress has been implicated in over a hundreds of disease states which range from arthritis, connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome [12]. Antioxidants have been shown to prevent oxidative damage caused by free radicals and reactive oxygen species (ROS) [13-14]. However, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone used in foods for preventing lipid peroxidation have been reported to produce toxicity [15] and carcinogenicity [16]. Therefore a few natural products have attracted attention because of their ability to remove free radicals [17]. Natural antioxidants such as flavonoids, tannins, coumarins, curcuminoids, xanthons, phenolics, and terpenoids are found in various plant products such as fruits, leaves, seeds, and oils [18-22].

The bark and leaves of *Cinnamomum* spp. are commonly used as spices in the home kitchen and their distilled essential oils or synthetic analogs are used as flavoring agents in the food and beverage industry [23]. This plant has been used in Ayurvedic (Indian traditional medicine) and other medicinal traditions in Asia. Cinnamon has been used for treatment of diarrhea, stomach upset, against respiratory ailments and externally as a skin antiseptic and rubefacient [9, 24-25]. Antifungal and antibacterial principles present in essential oil are effective in preventing food spoilage [26-28]. Cinnamon oil has proven to be particularly effective against some species of toxicogenic fungi [25] and against respiratory tract pathogens, including species belonging to the genera *Aspergillus, Candida, Cryptococcus,* and *Histoplasma* [24, 29]. Phytochemical moieties in *Cinnamomum* spp. possess antioxidant action that may prove beneficial against free radical damage to cell membranes [19].

A cursory survey of the literature reveals that no systematic study has been conducted regarding application of phytochemicals extracted from *C. zeylanicum* as antifungal and antioxidant agents. The objectives of this study were to prepare phytochemical rich extract fractions from *C. zeylanicum* bark and leaves; to evaluate their antifungal activity against *Aspergillus spp., Penicillium spp. and Candida albicans* as well as to assess their antioxidant activity.

2. Materials and Methods

2.1. Plant Material

The bark and leaf samples of *C. zeylanicum* were collected from Forest Research Institute, Dehradun, Uttarakhand, India. Freshly collected plant samples were shade-dried at room temperature for 10-15 days. Dried plant materials were separately crushed and ground into fine powder with mortar and pestle.

2.2.Preparation of extracts

Powdered plant materials were sequentially extracted with several solvents in a Soxhlet apparatus for 6-8 h as described elsewhere [10]. The solvents used for extraction included petroleum ether (PE), benzene (BZ), chloroform (CH), ethyl acetate (EA), acetone (AC), ethanol (ET) and water (AQ). Respective extracts were filtered and dried under reduced pressure. The dried extracts were preserved at 4^0C until used.

2.3.Test fungi

Aspergillus flavus, A. fumigatus, A. niger, Penicillium spp. and *Candida albicans* were isolated from soil on potato dextrose agar (PDA) plates. These moulds were grown and maintained on PDA slants at 28 ± 1°C. Following incubation for five days, the cultures were either utilized for test or stored at 4 ± 1°C for further use. The organisms were subcultured once in every fifteen days and the purity of the cultures was checked regularly under microscope.

2.4.Antifungal assay

For the study of antifungal activity, stock extract solutions were prepared in DMSO. Fungal growth inhibition was evaluated at an extract concentration of 1000 µg/ml. Cup well assay method was used to study the antifungal efficacy of *C. zeylanicum* extracts [10, 30]. Briefly, 0.1 ml of fungal broth culture was spread on the surface of PDA plates. About 50 µl of each extract solution was poured in separate wells with the help of micropipette. Pure DMSO was used as control. All the operations were carried out aseptically in the laminar chamber. Plates were incubated at 28 ± 1°C for 5 days. Antifungal activity was determined by measuring diameter of the zone of inhibition (ZOI) surrounding wells. The tests were performed in triplicate and the results were averaged.

2.5. Determination of Minimum fungicidal concentration (MFC) of the extracts

Malt extract having various concentrations of plant extracts (300, 500, 750 and 1000µg/ml) was prepared. Tubes containing 10 ml of above solution were inoculated with 0.1 ml of different fungal spore suspensions separately and were incubated at 28^0C ± 1^0C for 5-7 days. The lowest concentration of the plant extracts that did not permit any visible growth of the inoculated test fungi in the broth medium was regarded as the minimum inhibitory concentration in each case. Control experiments were performed without the plant extracts. The contents of the tubes showing no visible fungal growth or turbidity were further subcultured on freshly prepared PDA plates to determine if the inhibiton was reversible or permanent to assess the fungicidal efficacy of the extracts. The plates were incubated at 28^0C ± 1^0C for 5-7 days. All the tests were done in three replicates. The lowest concentration of the extract that did not produce any fungal growth on the solid medium was regarded as MFC value [5].

2.6.Determination of antioxidant activity by reducing power assay

Antioxidant activity was determined by the reducing power assay [21] with minor modification. One milliliter of different concentrations of cinnamon bark and leaf extracts (200, 400, 600, 800 and 1000 µg/ml in DMSO) was mixed with 1 ml methanol in a 10 ml test tube. It was followed by addition of 2.5 ml potassium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (10 g/l). The mixture was incubated at 50^0C for 20 min. At the end of incubation 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 5000g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water followed by addition of 0.5 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Ascorbic acid was used as reference material. All tests were performed in triplicate. Increase in absorbance of the reaction mixture indicated the increased reducing power of the samples.

2.7.Statistical analysis

All the experiments were performed in triplicate. For antifungal assay the results were averaged. However, for antioxidant assay experimental results were expressed as mean ± SEM of three parallel measurements. The diagrams were prepared using Graphpad Prism software.

3.Results

3.1.Antifungal activity of bark extracts

C. zeylanicum bark extracts were evaluated for antifungal activity against test fungi namely *A. fumigatus, A. niger, A. flavus, Penicillium spp.,* and *C. albicans* at an extract concentration of

1000 µg/ml. The results are shown in Table 1. *C. albicans* was inhibited by all the extracts showing inhibition zone sizes in the range of 8-16 mm while *A. niger* showed resistance to most of the extracts. In general BZ extract was least effective while rest of the extracts demonstrated moderate to high antifungal potential. All the test fungi exhibited sensitivity to polar fractions viz., AC, ET and AQ extract fractions. Non polar fractions (PE, BZ and CH extracts) did not show activity against most of the fungi.

Table 1. Fungicidal activity of *C. zeylanicum* bark extracts

Fungal strains	Extracts					
	PE	BZ	CH	AC	ET	AQ
A. fumigatus	-	-	-	10	13	14
A. flavus	-	-	-	14	15	9
A. niger	-	-	-	8	10	8
Penicillium spp.	8	8	-	10	12	13
C. albicans	15	8	10	12	13	16

Zone of inhibition is shown in mm. Antifungal activity of *C. zeylanicum* bark extracts was evaluated at a concentration of 1000 µg/ml. The extracts were prepared in petroleum ether (PE), benzene (BZ), chloroform (CH), acetone (AC), ethanol (ET) and distilled water (AQ) as described in methods section.

3.2.Antifungal activity of leaf extracts

Results of antifungal activity of *C. zeylanicum* leaf extracts are depicted in Table 2. Low to moderate activity was observed against test fungi. Polar fractions (AC, ET and AQ extracts) exhibited comparatively better fungicidal activity as compared to non polar fractions. Among all the extracts tested, ET extract demonstrated appreciable fungicidal activity against most of the fungal cultures showing 10-14 mm of zone of inhibition (ZOI). C. albicans showed sensitivity towards all the test extracts. PE, BZ and CH extracts were least effective. CH extract showed activity against only two fungi, namely, A. niger and C. albicans with ZOI 8 mm and 10 mm, respectively.

Table 2. Fungicidal activity of *C. zeylanicum* leaf extracts

Fungal strains	Extracts						
	PE	BZ	CH	EA	AC	ET	AQ
A. fumigatus	8	8	-	10	11	12	10
A. flavus	8	8	-	9	12	12	10
A. niger	-	8	8	-	8	12	8
Penicillium spp.	8	8	-	9	12	10	9
C. albicans	8	8	10	12	10	14	10

Zone of inhibition is shown in mm. Antifungal activity of *C. zeylanicum* leaf extracts was evaluated at a concentration of 1000 µg/ml. The extracts were prepared in PE, BZ,CH, EA, AC, ET and distilled water (AQ) as described in methods section.

3.3.Minimum fungicidal concentration (MFC) of extracts

The tests were performed at four different concentrations of each extract (300, 500, 750 and 1000 µg/ml). Results of bark extracts are shown in Table 3. The MFC values ranged between 300-1000 µg/ml. MFC of AC, ET and AQ extract fractions against A. niger was high (1000 µg/ml). Highest antifungal efficacy was obtained for AC, ET and AQ extracts against *A. flavus* (MFC 300 µg/ml). Similar MFC values (300 µg/ml) were recorded for AQ extract against *Penicillium spp.* and *C. albicans.* MFC for AC and ET extracts against *A. fumigatus, Penicillium spp.* and *C. albicans* was 500 µg/ml.

The MFC of *C. zeylanicum* leaf extracts was evaluated for the extracts which inhibited the fungal cultures at initial test concentration. The values were found in the range of 300-1000 µg/ml. It can be observed from table 4 that MFC for PE, BZ and CH extracts was very high (1000 µg/ml). Appreciable fungicidal activity (MFC 300-500 µg/ml) was recorded in EA, AC and ET extracts. The best antifungal efficacy was observed in ET extracts against test fungi with MFC about 300µg/ml. Water (AQ) extracts produced maximal inhibition (MFC 500 µg/ml) against A. *fumigatus* and *C. albicans.*

Table 3. Minimum Fungicidal Concentration (MFC) of *C. zeylanicum* bark extracts.

Fungi	Extracts					
	PE	BZ	CH	AC	ET	AQ
A. fumigatus	-	-	-	500	500	500
A. flavus	-	-	-	300	300	300
A. niger	-	-	-	1000	1000	1000
Penicillium spp.	1000	1000	-	500	500	300
C. albicans	500	1000	1000	500	500	300

MFC values are shown in µg/ml. Abbreviations: PE - Petroleum ether, BZ Benzene, CH Chloroform, AC Acetone, ET Ethanol, AQ Aqueous*) .

Table 4. Minimum Fungicidal Concentration (MFC) of *C. zeylanicum* leaf extracts

Fungi	Extracts						
	PE	BZ	CH	EA	AC	ET	AQ
A. fumigatus	1000	1000	-	500	300	300	500
A. flavus	1000	1000	-	500	500	300	1000
A. niger	-	1000	1000	-	1000	300	1000
Penicillium spp.	1000	1000	-	500	300	500	1000
C. albicans	1000	1000	1000	500	500	300	500

MFC values are shown in µg/ml. Abbreviations: PE - Petroleum ether, BZ Benzene, CH Chloroform, EA Ethyl acetate, AC Acetone, ET Ethanol, AQ Aqueous.

3.4.Antioxidant activity of C. zeylanicum extracts

Antioxidant activities of the extracts were assayed by reducing power assay. Results of activity of bark extracts are presented in figure 1. Higher absorbance values indicated higher reducing power. The reducing power of the extracts increased with increasing concentration of extracts exhibiting dose dependent response. Among the fractions assayed, polar extracts of *C. zeylanicum* showed the strongest activity. Three extracts viz., AC, AQ and ET produced potential reducing power. AC extract of the bark exhibited highest activity followed by AQ at all the test concentrations. As compared to standard antioxidant ascorbic acid, both the extracts produced more than 50% reducing power at highest test concentration of extracts. ET fractions showed comparatively lower reducing power. The non polar fractions accounted for very low activities.

Reducing power of *C. zeylanicum* leaf extracts are presented in figure 2. Leaf extracts accounted for lower reducing power as compared with the activities of bark extracts. The antioxidant activities slowly increased with increasing concentration of extracts. At higher concentrations (800 and 1000 µg/ml) AQ extract exhibited higher reducing power. The order of reducing power. The order of reducing power laz leaf extracts was AQ>CH>ET>EA>AC>PE=BZ.

Figure 1. Reducing power of *C. zeylanicum* bark extracts. The bark extracts were prepared in (1) petroleum ether (PE),(2) benzene (BZ), (3) chloroform (CH), (4) acetone (AC), (5) etanol (ET) and (6) water (AQ) as described in Methods section. Ascorbic acid (Std) was used as standard antioxidant for comparison. Five concentrations (200,400,600, 800 and 1000 g/ml have been used to evaluate the antioxidant activity of extracts as mentioned inMethodssection.

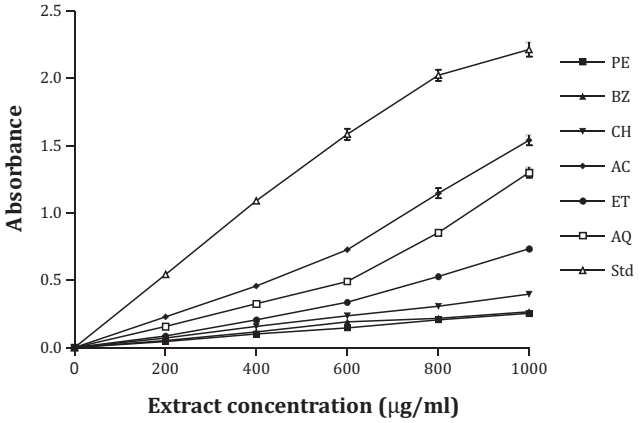
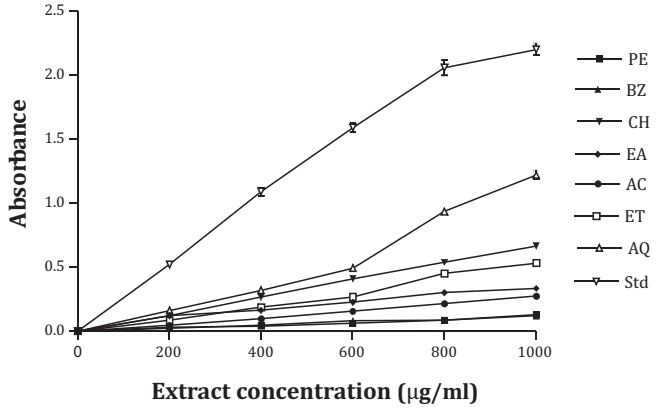


Figure 2. Reducing power of C. zeylanicum leaf extacts. The leaf extracts were prepared in (1) PE, (2) BZ, (3) CH, (4) ethyl acetate (EA), (5) AC, (6) ET and (7) AQ as described in Methods section. Ascorbic acid (Std) was used as standard antioxidant for comparison. Other conditions were same as described in Fig.1.



4.Discussion

Antimicrobial susceptibility and antioxidant testing remains an area of intense interest. Developments in this area provide new leads for drug discovery. Number of reports is available showing efficacy of *C. zeylanicum* essential oils as antimicrobial agents but data regarding use of extracts as antifungal agents are rare [8]. The present work demonstrates the fungicidal efficacy of C. zeylanicum bark and leaf extracts against *A. fumigatus, A. niger, A. flavus, Penicillium spp. and C. albicans.* It seems that antifungal principles are mostly concentrated in polar fractions as evident from the experimental findings. MFC (minimum fungicidal concentration) of polar extract fractions against test fungi were found in the range of 300-1000 µg/ml showing appreciable inhibitory potential in some of the extracts.

Available reports tend to show that secondary metabolites such as alkaloids, flavonoids, tannins and other compounds of phenolic nature are the responsible compounds for the antimicrobial activities in higher plants [31-32]. Phytochemical screening of the *C. zeylanicum* has revealed that extracts from bark and leaves possess at least three to four of the following classes of secondary metabolites: phenols, flavonoids, terpenoids, tannins, alkaloids and saponins [8]. Therefore, the presence of these phytochemicals could to some extent justify the observed antifungal activities in the current study. These results are in agreement with many studies realized on other plant species belonging to the euphorbiaceae [32] and asteraceae [33] attributing antimicrobial activities to the presence of secondary metabolites.

The experimental findings indicate that antifungal substance within this plant seems to be more prominently present in the bark as compared to leaves. The difference could be attributed to the presence of variable amounts of bioactive secondary metabolites in different parts of the plant. The composition of these secondary metabolites in turn varies from species to species, climatic conditions, and the physiological state of developments of the plants [10].

Spice plants, being rich sources of essential oil, have been shown to possess strong antifungal activity against fluconazole-resistant and fluconazole susceptible Candida spp. namely C. albicans, *C. tropicalis, C. glabarata and C. krusei* [34]. The inhibitory effects of spices are mostly due to the volatile oils present in their composition. *C. zeylanicum* has been reported to possess fungitoxic activities against storage fungi. The vapour emitted by the bark has been shown to inhibit the mycelial growth of A. flavus and A. niger completely [35]. The organic and aqueous extracts obtained from *C. zeylanicum* bark and leaves have demonstrated potentential antifungal activity by inhibiting spore germination in two dematiacious moulds, *Alternaria solani and Curvularia lunata* [8].

C. albicans, a dimorphic fungus, exhibited susceptibility to most of the extracts derived from *C. zeylanicum* bark and leaves. There are reports that fungicidal agents act against both morphogenetic transformation and the budding process. Antifungal agents with a high fungicidal potential, also have a high potential to block morphogenetic transformation against both the yeast and hyphal forms of *C. albicans* and this may be related to their fungicidal potential [4]. The fungicidal agents disrupt membrane [36] or cell wall integrity [37], and consequently inhibit the hyphal form at low concentrations. It has been reported that the less fungicidal agents which exert their antifungal action through inhibition of cytochrome P450 demethylase [38], squalene epoxidase [39], and RNA and DNA synthesis [36] respectively, tend to be less effective against morphogenetic transformation, suggesting that they preferentially inhibit the budding process.

The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with the evidence that increased hydroxylation results in increased toxicity [40]. In addition, some authors have found that more highly oxidized phenols are more inhibitory [41]. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins [13]. Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in cinnamon and clove oils. Eugenol is considered effective against both fungi and bacteria [13].

There is no practical, cost effective and non toxic method for preventing fungal deterioration of stored food product commodities. Therefore use of non-toxic edible substance to control fungal deterioration of stored grain and seeds is highly desirable. There are some reports on antimicrobial activity of *C. zeylanicum* against bacteria, viruses, moulds and yeasts. The results have ranged according to the microorganism and assayed product (essential oil, extracts, decoct, plant powder). Phytochemicals are small organic biomolecules generally hydrophobic and designated as naturally occurring antibiotics [42]. Antifungal property of phytochemicals could involve cytosolic hyperacidity, breakage of electrons transport chain, H+-ATPase inhibition, channels inhibition, intracellular and extracellular enzymes synthesis inhibition [43].

Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT), have restricted use in foods as these synthetic derivatives are suspected to be carcinogenic [44]. Therefore, the importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years.

Reducing properties of phytochemicals responsible for radical scavenging activities are generally associated with the presence of reductones [45]. The antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom [46]. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The experimental data obtained in the present work indicated marked antioxidant activity in some of the *C. zeylanicum* extracts. It could be attributed to the presence of polyphenols [8], which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction [44]. Similarly reducing power of tannins have also been shown to prevent liver injury by inhibiting the formation of lipid peroxides [47]. Therefore reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity [48].

Aqueous, acetone and ethanol extracts demonstrated high antioxidant activity in reducing power assay. A positive correlation was observed between the antioxidant and antifungal activities of these extracts. The results indicate that bark and leaf of *C. zeylanicum* are rich sources of the antioxidants exhibiting higher activity. This could be attributed to the presence of differential amount of phenolics, flavonoids, tannins and terpenoids etc. in extracts [49]. The difference in the antioxidant activity of the bark and leaf may be accounted for by their different phytochemical composition. Thus, our findings indicate that the selective extraction of antioxidant from natural sources by appropriate solvent is very important in obtaining fractions with high antioxidant activity. There are reports that polyphenols, particularly flavonoids, which are widely distributed in the plant kingdom, and are present in considerable amounts in fruits, vegetables, spices, medicinal herbs, and beverages, have been used to treat many human diseases, such as diabetes, cancer and coronary heart disease [49]. Moreover, flavonoids have been shown to exhibit the antioxidative, antiviral, antimicrobial and anti-platelet activities [50].

Based on accumulative evidence, natural antioxidants have recently attracted considerable attention for their presumed role in protecting the human body against a large number of infectious and degenerative diseases. Growing experimental evidence has suggested that antioxidants can improve a wide range of cell biological functions by virtue of their radical scavenging activity [51].

5.Conclusions

The results of the present work indicate the presence of compounds possessing antifungal and antioxidant activity in *C. zeylanicum* extracts with bark as an enriched source exhibiting higher activity as compared to leaves. The low MFC values of a few extracts against some of the most important food poisoning and spoilage organisms reveals an exciting potential for application in food systems. Furthermore, higher antioxidant activity of these compounds will be an added advantage in providing a safe and natural alternative to chemical preservatives.

6.References

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